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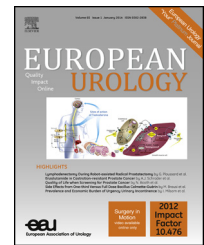
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From Lab to Clinic

Carbonic Anhydrase 9 Expression Increases with Vascular Endothelial Growth Factor–Targeted Therapy and Is Predictive of Outcome in Metastatic Clear Cell Renal Cancer

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Abstract

Background: There is a lack of biomarkers to predict outcome with targeted therapy in metastatic clear cell renal cancer (mccRCC). This may be because dynamic molecular changes occur with therapy.

Objective: To explore if dynamic, targeted-therapy-driven molecular changes correlate with mccRCC outcome.

Design, setting, and participants: Multiple frozen samples from primary tumours were taken from sunitinib-naïve ($n = 22$) and sunitinib-treated mccRCC patients ($n = 23$) for protein analysis. A cohort ($n = 86$) of paired, untreated and sunitinib/pazopanib-treated mccRCC samples was used for validation. Array comparative genomic hybridisation (CGH) analysis and RNA interference (RNAi) was used to support the findings.

Intervention: Three cycles of sunitinib 50 mg (4 wk on, 2 wk off).

Outcome measurements and statistical analysis: Reverse phase protein arrays (training set) and immunofluorescence automated quantitative analysis (validation set) assessed protein expression.

Results and limitations: Differential expression between sunitinib-naïve and treated samples was seen in 30 of 55 proteins ($p < 0.05$ for each). The proteins B-cell CLL/lymphoma 2 (BCL2), mutL homolog 1 (MLH1), carbonic anhydrase 9 (CA9), and mechanistic target of rapamycin (mTOR) (serine/threonine kinase) had both increased intra-tumoural variance and significant differential expression with therapy. The validation cohort confirmed increased CA9 expression with therapy. Multivariate analysis showed high CA9 expression after treatment was associated with longer survival (hazard ratio: 0.48; 95% confidence interval, 0.26–0.87; $p = 0.02$). Array CGH profiles revealed sunitinib

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was associated with significant CA9 region loss. RNAi CA9 silencing in two cell lines inhibited the antiproliferative effects of sunitinib. Shortcomings of the study include selection of a specific protein for analysis, and the specific time points at which the treated tissue was analysed.

Conclusions: CA9 levels increase with targeted therapy in mcrRCC. Lower CA9 levels are associated with a poor prognosis and possible resistance, as indicated by the validation cohort.

Patient summary: Drug treatment of advanced kidney cancer alters molecular markers of treatment resistance. Measuring carbonic anhydrase 9 levels may be helpful in determining which patients benefit from therapy.

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1. Introduction

Vascular endothelial growth factor (VEGF)-targeted tyrosine kinase inhibitor (TKI) therapy is established as first-line therapy in metastatic clear cell renal cancer (mcrRCC) [1]. Clinical benefit with sunitinib varies among mcrRCC patients. While there are a number of prognostic clinical factors, there are presently few validated molecular means of improving prognosis or prediction of response of mcrRCC patients to targeted therapies [2]; the 2012 report of serum interleukin-6 predicting response to pazopanib is an exception [3]. This lack of predictive ability is in contrast to numerous other tumour types, such as chronic myeloid leukaemia and breast cancer, in which protein expression and mutation analysis can be used to predict response and treatment failure [4,5].

Analysis of molecular markers from single tumour tissue samples taken at baseline in mcrRCC has failed to identify predictive biomarkers associated with response to sunitinib [6]. We hypothesise that dynamic changes occur to biomarker expression with VEGF-targeted therapy, and only tissue taken later in the course of treatment can predict drug activity. Therefore, by analysing protein expression from VEGF-treated and untreated renal cancer tissue, it may be possible to identify and validate protein biomarkers.

In this work, we compared the expression of 55 key proteins in nephrectomy tumour samples from patients with mcrRCC who were treated with sunitinib prior to nephrectomy or were sunitinib-naïve at the time of surgery. Extensive intratumoural heterogeneity (ITH) has been demonstrated in mcrRCC [7,8]. ITH is likely to hamper biomarker research. To address ITH in this study, lysates were taken for multiple, spatially separate areas of each primary tumour. We attempted to identify not only biomarkers that significantly change with VEGF-targeted therapy, but also those that demonstrated increased protein variance with therapy. To confirm these findings, a validation cohort was used, consisting of paired untreated and anti-VEGF TKI-treated samples ($n = 86$) taken from previously untreated mcrRCC patients enrolled in three clinical trials. To further explore the cause and relevance of changes in protein expression, array comparative genomic hybridisation (aCGH) was used to identify relevant chromosomal changes, while RNA interference (RNAi) in RCC cell lines addressed the functional relevance of significant changes.

2. Methods

2.1. Cell lines

See the Supplement for cell-line details.

2.2. Patient samples

Fresh frozen primary ccRCC tissue was obtained from the nephrectomy samples of 22 sunitinib-naïve mcrRCC patients as part of the Scottish Collaboration On Translational Research into Renal Cell Cancer study (UK Clinical Research Network identifier: 12229). Tissue was also obtained from 27 mcrRCC patients treated with three cycles of presurgical sunitinib (18 wk) as part of the Upfront Sunitinib (SU011248) Therapy Followed by Surgery in Patients with Metastatic Renal Cancer: a Pilot Phase II Study (SuMR; ClinicalTrials.gov identifier: NCT01024205). Tissue from four of these patients was entirely necrotic, leaving 23 patients with adequate tissue for analysis (Table 1). These two sample cohorts made up the test sample set.

A tissue microarray (TMA), with the paraffin-embedded tissue from matched, pretreatment primary tumour biopsy tissue and post-treatment nephrectomy tumour tissue from the same patients ($n = 86$), was used as a validation sample set (TMA construction details are shown in the Supplement). This tissue came from three prospective studies—the SuMR study, the Phase II Study Investigating Upfront Pazopanib In Metastatic Renal Cancer Renal Cancer (Panther) (ClinicalTrials.gov identifier: NCT01512186), and the Patient Preference Study of Pazopanib Versus Sunitinib in Advanced or Metastatic Kidney Cancer (ClinicalTrials.gov identifier: NCT01064310)—and included patients treated with sunitinib and pazopanib. Patients were followed up according to standard guidelines with cross sectional imaging performed every 12 weeks. Outcome data were recorded. All studies underwent ethics approval prior to commencement.

Each piece of fresh frozen tumour tissue was mapped and separated into small pieces (about 1 cm^3) from which lysates were created. A frozen section was performed (M.O.) on each 1-cm^3 piece of tissue to confirm the presence of viable ccRCC and also for grading. Where possible, we tried to obtain a minimum of four protein/DNA lysates per patient.

Table 1 – Patient demographics, pathology details, and clinical outcomes of test and validation patient cohorts from which there was adequate tumour tissue for molecular analysis

Cohort	Test cohort: total	Test cohort: sunitinib naïve	Test cohort: sunitinib treated	Validation cohort
Patients, no.	45	22	23	86
Age, yr, median (IQR) *	66.0 (58.9–73.0)	67.7 (59.1–73.3)	63.0 (56.0–73.0)	61 (51.0–66.3)
Male patients, no. (%) *	30 (67)	14 (64)	16 (70)	66 (77)
Fuhrman grade, no. (%) **				
I	0	0	0	0
II	14 (31)	4 (18)	10 (43)	32 (37)
III	22 (49)	10 (45)	12 (52)	31 (36)
IV	9 (20)	8 (36)	1 (4)	14 (16)
Missing data	0	0	0	9 (10)
Stage, no. (%) ***				
T1	1 (2)	0	1 (4)	9 (10)
T2	6 (13)	2 (9)	4 (17)	21 (24)
T3	33 (73)	20 (91)	13 (57)	37 (43)
T4	5 (11)	0	5 (22)	14 (16)
Missing data	0	0	0	5 (6)
VHL status, no. (%) *				
Mutation	32 (71)	17 (77)	15 (65)	NA
Wild type	13 (29)	5 (23)	8 (35)	
Metastatic sites, no. (%) *				
1	21 (47)	13 (59)	8 (35)	28 (32)
2	18 (40)	7 (32)	11 (48)	34 (40)
3	6 (13)	2 (9)	4 (17)	24 (28)
Heng classification, no. (%) *				
Intermediate	25 (56)	14 (64)	11 (48)	51 (59)
Poor	18 (40)	6 (27)	12 (52)	35 (41)
Missing data	2 (4)	2 (9)	0	0
First-line TKI, no. (%) ^	N/A	11 (50)	N/A	N/A
Overall survival, mo, median (IQR) *	16.0 (9.1–26.1)	12.3 (7.0–20.0)	23.0 (13.6–30.0)	18 (14.7–23)

IQR = interquartile range; VHL = von Hippel-Lindau; NA = not available; TKI = tyrosine kinase inhibitor; N/A = not applicable.

P values comparing sunitinib-naïve and sunitinib-treated patients: * $p > 0.05$, ** $p = 0.02$; *** $p = 0.04$.

^ Number of sunitinib-naïve patients who had postnephrectomy TKIs.

2.3. Reverse phase protein arrays

Protein extraction and reverse phase protein array (RPPA) spotting and protein quantification were performed as described previously [9,10]. Protein expression levels from RC124 and human umbilical vein endothelial cell lines were used as references on each RPPA slide. Batch effects across the three RPPA slides per marker were mitigated using ComBat [11] and data were normalised using variable slope normalisation [12].

RPPA was used to evaluate 58 proteins. These proteins were relevant in RCC pathogenesis or sunitinib response and belonged to the following functional groups: cell cycle, apoptosis, protein kinases, angiogenesis, cell adhesion, PI3K pathway, epithelial-to-mesenchymal transition, met proto-oncogene/hepatocyte growth factor and mismatch repair. There was no signal detected for three of the proteins (Ki67, FLT3, and phospho-Jak2). As such, 55 proteins were analysed in this study (antibodies are detailed in Supplemental Table 1).

2.4. Automated quantitative analysis of immunofluorescence

Immunofluorescence and automated quantitative analysis (AQUA) analysis were performed on the validation cohort, using methods previously described [13,14].

To correct for any bias due to the separation of pre- and post-treatment samples on unique TMAs, AQUA results for the matched tissue samples on each TMA were median

normalised prior to analysis of significance by the Wilcoxon matched-pairs test. X-tile was used for determining the cut-off for defining high and low protein expression in the primary tumour [15].

2.5. Array comparative genomic hybridisation

DNA extraction from fresh frozen tissue and formalin-fixed, paraffin-embedded specimens was carried out using the DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. aCGH hybridisation and analysis was carried out as described by Gerth-Kahlert et al. [16], using the Roche NimbleGen 12 × 135K whole-genome array (Roche NimbleGen, Madison, WI, USA).

The CGH-segMNT module of NimbleScan was used for the analysis, with a minimum segment length of five probes and an averaging window of 130 kb. NimbleGen arrays were positionally annotated based on hg19 genomic coordinates, and log ratio data were preprocessed in R, as previously described [17]. Briefly, array data were normalised with print tip Loess from the limma package in R to produce normalised log ratios, filtered to remove outliers based on 1 mean absolute deviation of each probe from its immediate genomic neighbours, and smoothed with a circular binary segmentation algorithm from the DNACopy package. Thresholds for smoothed log ratios were then determined for gain/loss (± 0.1) and amplification/deletion (± 0.45) to identify contiguous copy number aberrations

containing at least 15 consecutive probes. Further details of aCGH analysis are given in the Supplement.

2.6. RNA interference experiments

The human RCC cell lines CAKI-2 (wild-type von Hippel-Lindau [VHL]) and RCC11 (a VHL mutant) were transfected

with a nontargeting, control, short interfering RNA (siRNA) (5'-CATGCCTGATCCGCTAGTC-3') or carbonic anhydrase 9 (CA9) siRNA (5'-GAGGAGGATCTGCCAGT-GAA-3') (Qiagen, Manchester, UK). Twenty-four hours after transfection, cells were treated with either 0.01% dimethyl sulfoxide or 4 μ M sunitinib and cell viability was assessed after 5 d using the Cell Titer Glo assay (Promega,

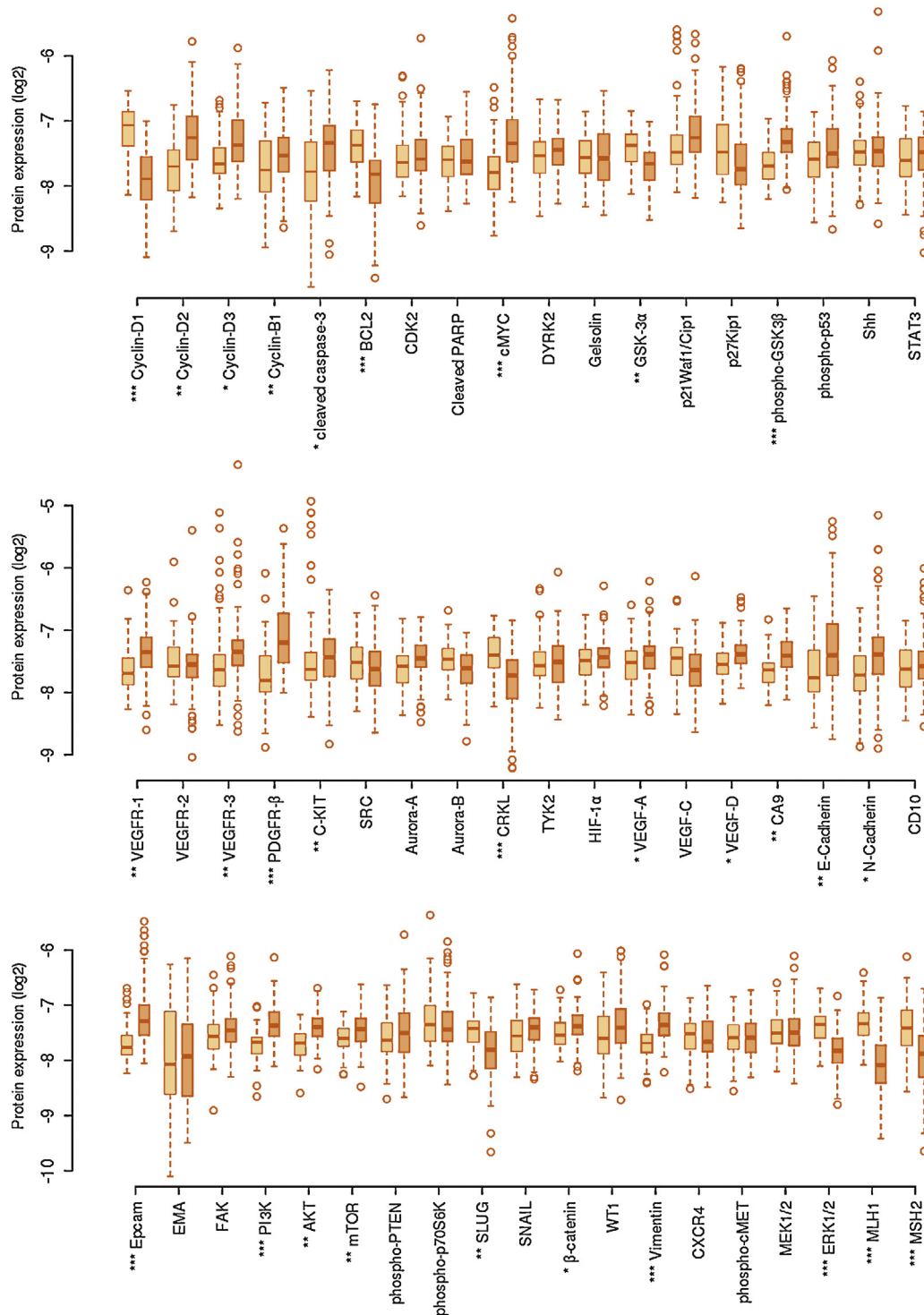


Fig. 1 – Reverse phase protein array (RPPA) differential protein expression results. Box-and-whisker plot showing differential expression analysis of 55 proteins evaluated by RPPA in sunitinib-naïve (light orange) and treated (dark orange) metastatic clear cell renal cell carcinoma samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Southampton, UK). The Supplement presents further RNAi methodology.

2.7. Statistical analysis

Differential expression was assessed per protein between sunitinib-treated and sunitinib-naïve tumours by application of the *t* test where normality and homoscedasticity assumptions were satisfied; otherwise, the nonparametric Mann-Whitney U test was used. The F test was used to assess intratumoural variances within an analysis of variance framework for those proteins for which assumptions of normality and homoscedasticity (within group) were met. Appropriate false-discovery rate correction was applied to all *p* values [18]. Further details on the assessment of intratumoural variance are given in the Supplement.

Overall survival was estimated using Kaplan-Meier methods, with differences assessed using the log-rank test. Multivariate analysis was performed using Cox regression. SPSS v.20 (IBM Corp, Armonk, NY, USA) or R were used for all statistical analyses.

3. Results

3.1. Patient demographics

The key patient characteristics and treatment outcomes were comparable for patients in the test set who were not treated with sunitinib prior to a cytoreductive nephrectomy and those patients who had sunitinib therapy prior to nephrectomy (Table 1). Of the 45 patients included, 44 had multiple samples taken (median: 4 regions; range: 2–10 regions).

3.2. Effect of sunitinib treatment on protein expression assessed by reverse phase protein array

There were significant differences in protein expression between the treated and untreated samples for 30 of the 55 proteins evaluated in the test set (Fig. 1). Of particular note were four proteins that had both significant differential expression and significantly increased intratumoural variance after sunitinib: B-cell CLL/lymphoma 2 (BCL2), mutL homolog 1 (MLH1), CA9, and mechanistic target of rapamycin (serine/threonine kinase) (mTOR) ($p < 0.05$ for each) (Fig. 2a).

3.3. Automated quantitative analysis results from the validation cohort

BCL2, MLH1, CA9, and mTOR protein expression was evaluated using in situ staining and AQUA of the validation TMA (paired treated and untreated samples from the same patient [$n = 86$]). This analysis revealed that of these four proteins, only CA9 was significantly differentially expressed (increased) with treatment ($p = 0.01$) (Fig. 2b). High expression of CA9 in sunitinib-treated tissue was associated with good overall survival (OS) (hazard ratio [HR]: 0.26; 95% confidence interval [CI], 0.11–0.61; log rank test $p = 0.001$) (Fig. 2c). Results from the multivariate analysis, which

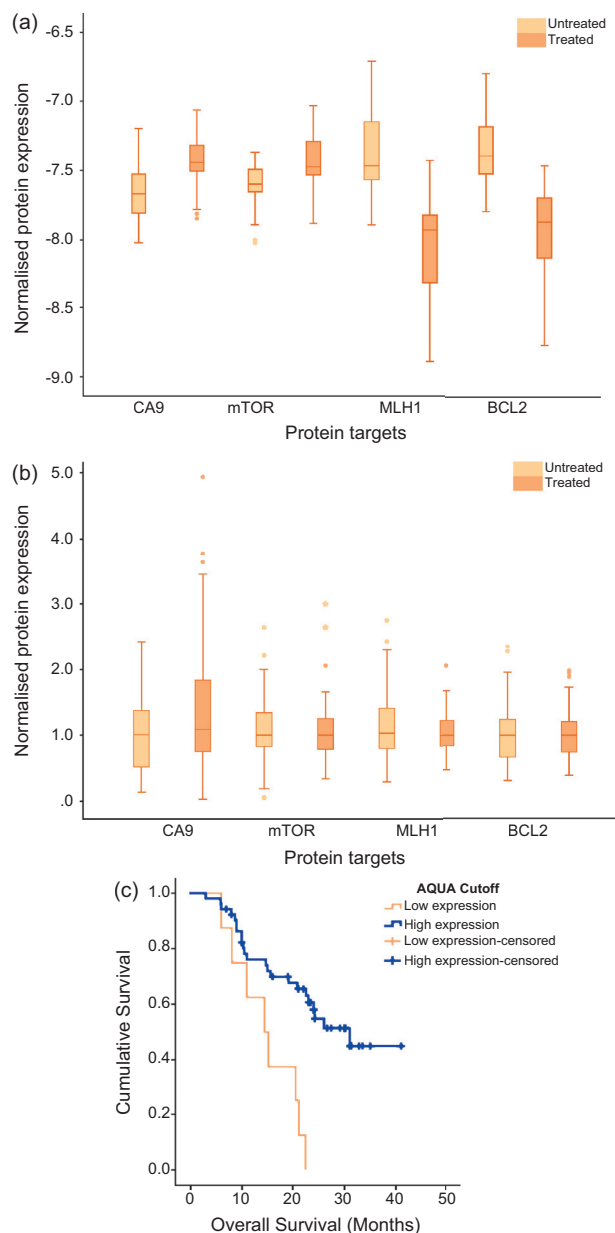


Fig. 2 – Results for significantly variable and differentially expressed proteins in test and validation samples from sunitinib-naïve and treated patients. (a) Box-and-whisker plot showing test set reverse phase protein array differential expression results of four key proteins. Medians and interquartile ranges are shown. (b) Box-and-whisker plot showing automated quantitative analysis (AQUA) evaluated protein expression of four key proteins using a validation cohort of 61 sunitinib-treated and 25 pazopanib-treated and untreated paired metastatic clear cell renal cell carcinoma samples. Of the four proteins, carbonic anhydrase 9 (CA9) was the only one with significant differential protein expression ($p = 0.01$). Medians and interquartile ranges are shown. (c) Kaplan-Meier curve showing relationship of CA9 protein expression determined by AQUA in situ analysis (low vs high, as determined using X-tile [15]) in sunitinib/pazopanib-treated patients to overall survival (hazard ratio: 0.260; 95% confidence interval, 0.111–0.608; $p = 0.001$).

included a number of prognostic factors (ie, Heng prognostic score, Fuhrman grade, T stage at diagnosis, number of metastatic sites, age, and CA9 expression in nephrectomy ccRCC specimen) in the model showed that low Fuhrman

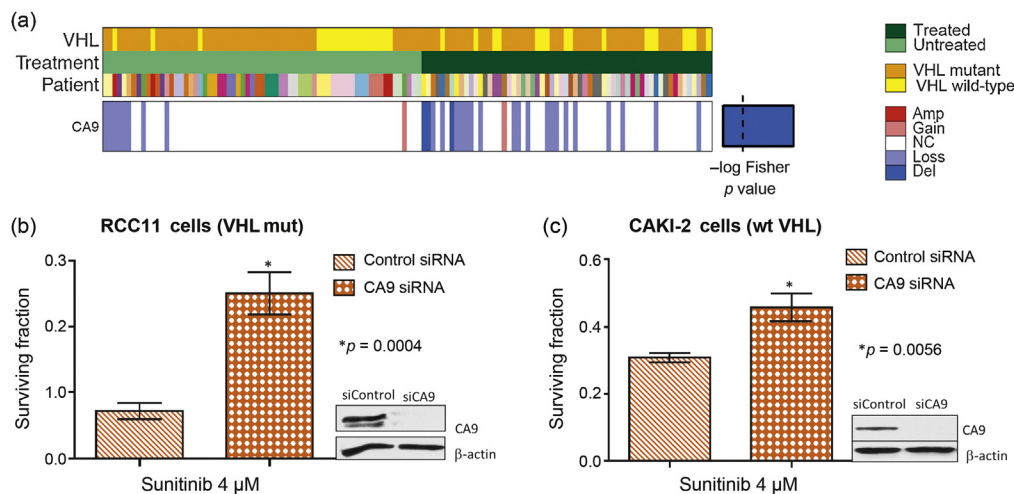


Fig. 3 – Array comparative genomic hybridization and RNA interference carbonic anhydrase 9 (CA9) results. (a) Heat map plotting gains (red) and losses (blue) of CA9. The right-hand bar represents the logarithm of the odds score ($-\log_{10}$) of the adjusted p value (Fisher test), the dashed line represents $p = 0.05$. There were significantly more losses in the treated samples relative to the untreated patient samples ($p = 0.002$). Supplemental Figure 1 provides further description of regional chromosomal changes and Supplemental Figure 2 provides details of the genome-wide changes in gains and losses following sunitinib therapy. (b) RCC11 and (c) CAKI-2 human renal cell carcinoma transfected with either control or CA9 short interfering RNA (siRNA), followed by sunitinib treatment and cell viability analysis 5 d later. Error bars represent standard errors of the mean. To confirm silencing, cell lysates from RCC11 and CAKI-2 siRNA transfected cells were analysed by western blotting using CA9 and β -actin-specific antibodies, as indicated. VHL = von Hippel-Lindau; Del = deletion; mut = mutant; NC = no change; wt = wild-type.

grade (HR: 0.51; 95% CI, 0.30–0.89) and high CA9 expression at nephrectomy (HR: 0.48; 95% CI, 0.26–0.87), were associated with a good OS. Full results are reported in Supplemental Table 2.

3.4. Array comparative genomic hybridisation analysis

DNA from the test set of samples from sunitinib-naïve and sunitinib-treated patients was used for aCGH analysis to compare chromosomal aberrations. The total number of aberrations was significantly greater in treated samples. Comparisons of gains, losses, amplifications, and deletions in sunitinib-treated and untreated samples revealed significantly greater levels of chromosomal losses in the region encoding CA9 in the treated samples (Fisher test $p = 0.002$) (Fig. 3a). Conversely, the increase in losses across the whole genome was not significant (Supplemental Fig. 2).

3.5. Functional analysis of CA9 using RNA interference in renal cancer cell lines

Results from both renal cancer cell lines (CAKI-2 and RCC11) showed CA9 was successfully silenced with siRNA (Fig. 3b and 3c). The cell viability assay showed CA9 silencing inhibited the antiproliferative effects of sunitinib, regardless of cell-line VHL status. These results support the findings from the clinical tissue, where low levels of CA9 were associated with poor outcome from sunitinib therapy.

4. Discussion

In this work, we have demonstrated that VEGF-targeted therapy significantly alters the expression of a number of selected proteins despite protein ITH.

Four proteins—CA9, MLH1, mTOR, and BCL2—showed both significant changes in expression and increases intratumoural variance with sunitinib. These dynamically variable and changing proteins were chosen for further evaluation due to the likelihood of them being biologically relevant. Of these four proteins, CA9 upregulation revealed significant results in the validation cohort and functional cell-line work.

CA9 is a hypoxia-regulated transmembrane protein overexpressed in a number of cancers. It is usually associated with hypoxic stress and poor prognosis [19]. Extensive investigation has been performed in renal cancer, due to the frequency of CA9 overexpression and conflicting results regarding its prognostic value [19–21]. CA9 is overexpressed in the vast majority of ccRCC and has promise at a diagnostic level [20]; but paradoxically, high CA9 levels correlate with good outcomes in some studies [21]. Tumour samples in the pre-VEGF TKI era showed conflicting data on the prognostic value of baseline CA9 [21,22]. Prospective studies showed CA9 was not able to predict response to immune therapy [23]. Together, these data suggested that CA9 did not have a crucial predictive role in the era of immune therapy.

Biomarker studies in the era of VEGF-targeted therapy have failed to consistently show that high baseline CA9 protein levels are associated with a good outcome [24,25]. This may be because of the dynamic changes that occur with therapy and the use of archival tissue from a single time point for biomarker analysis. The work presented here shows that not only does targeted therapy increase the expression of CA9, but these changing levels are also prognostic. These findings were observed in both interpatient (unmatched test set) and, critically, the inpatient (matched sequential biopsy and nephrectomy validation set) samples.

This dynamic change in a prognostically important biomarker suggests the drive towards predictive biomarkers may be possible. Anti-VEGF therapy is associated with vasoconstriction and subsequent hypoxia [8]; therefore, the upregulation of CA9 with VEGF-targeted therapy could be a consequence of effective VEGF targeting. Indeed, Figure 1 shows that a number of VEGF- and hypoxia-associated markers are also affected by sunitinib (eg, VEGF receptor [VEGFR]-1, VEGFR-3, platelet-derived growth factor receptor- β , c-KIT, VEGF-A, VEGF-D), supporting this argument. Of note, hypoxia-inducible factor 1 α was not differentially expressed, most likely due to its short half-life, which was exceeded by the warm ischaemia time during sample acquisition.

Alternatively, CA9 may have more of a direct oncogenic effect as a reaction to VEGF-targeted therapy. The silencing of CA9 in renal cancer cell lines resulted in inhibition of the antiproliferative effects of sunitinib. While not conclusive, these in vitro findings support our clinical sample data. Together, these results suggest a role for CA9 in sunitinib activity and that CA9 may be relevant in the development of sunitinib resistance. Previous studies have shown that CA9 can affect cell adhesion and contact inhibition, demonstrating a role beyond simply a reaction to hypoxia [26,27]. The work presented here also shows that sunitinib treatment is associated with chromosomal changes to CA9. These chromosomal changes also point to a change in tumour DNA rather than simply a stromal reaction to hypoxia.

A biomarker identified after a specific period of therapy is of potential clinical use, providing patients are willing to have a repeated biopsy during treatment. The utility of repeated biopsy in practice is challenging. A randomised trial comparing continued therapy with a change in therapy in those patients who failed to gain a rise in CA9 level with therapy would test this biomarker prospectively.

There are several strengths to this work. Obtaining sequential tissue in metastatic renal cancer is challenging and this is, to our knowledge, the largest series available. Different techniques to measure protein expression were used in the training and validation set; furthermore, the chromosomal and in vitro work support the CA9 findings. However, there are a number of limitations of this study. First, the 55 proteins chosen for analysis may be subject to selection bias. Also, the initial biomarker testing was not performed in matched pairs from the same individual. Ideally, RPPA and aCGH would have been performed using matched samples from the same patient before and after sunitinib therapy; this was not feasible due to the amount of fresh frozen tumour tissue required to allow multilevel molecular analysis from multiple, spatially variant regions of the same tumours. However, the characteristics of the two test-set groups were similar and validation of the biomarkers occurred in paraffin-embedded matched pairs. Biomarker analysis took place at a specific time point with therapy and following a 2-wk break before nephrectomy, which may have influenced biomarker expression. Finally, the in vitro study does not assess the effect of sunitinib on the tumour vasculature [28], the main target of this

treatment, which is a limitation of the epithelial cell culture used in this study.

5. Conclusions

This study illustrates the dynamic changes to relevant proteins with anti-VEGF targeted therapies. Despite these dynamic changes and factoring in ITH, it was possible to identify and validate CA9 as an independent predictor of outcome following anti-VEGF targeted therapy. There were consistent dynamic changes to CA9 at chromosomal and protein levels. Together with the multivariate analysis and in vitro studies, these results suggest CA9 may have relevance to sunitinib resistance. CA9 modulation to overcome anti-VEGF therapy resistance may be a potential therapeutic area of investigation in the future.

Author contributions: Thomas Powles had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Stewart, Powles, Harrison, Overton.

Acquisition of data: Stewart, Laird, O'Mahony, O'Donnell, Berney, Rashid, Martin, Mullen, Nanda.

Analysis and interpretation of data: Stewart, Laird, O'Mahony, Eory, Lubbock, MacKay, Rashid, Martin, Bex, Overton, Harrison, Powles.

Drafting of the manuscript: Stewart, Powles.

Critical revision of the manuscript for important intellectual content: Harrison, Bex, Overton, Riddick, McNeill, Aitchison.

Statistical analysis: Stewart, O'Mahony, Eory, Lubbock, Overton, Powles.

Obtaining funding: Stewart, Laird, Powles, Harrison, Overton.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.eururo.2014.04.007>.

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